

## Moss seedling by sterile culture of plant tissue

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### Abstract of GB 2282822 (A)

Plant tissues of mosses, such as cut tissue pieces, gametophores, calluses, protonemata, spores, gemmae, subterranean stems or rhizoids, are cultured in a sterile medium, preferably comprising a monosaccharide or disaccharide, to generate seedlings (cultigen) corresponding to plumules. The seedlings may be sown onto a nursery at a desired colony density, thereby to produce a dense moss mat.

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## (54) Moss seedling by sterile culture of plant tissue

(57) Plant tissues of mosses, such as cut tissue pieces, gametophores, calluses, protonemata, spores, gemmae, subterranean stems or rhizoids, are cultured in a sterile medium, preferably comprising a monosaccharide or disaccharide, to generate seedlings (cultigen) corresponding to plumules. The seedlings may be sown onto a nursery at a desired colony density, thereby to produce a dense moss mat.

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The present invention relates to a seedling of moss (bryophyte) usable as a cultigen and a method of producing a dense 5 moss mat by using such seedlings, and more particularly to a moss seedling easily cultivated to a dense colony, and a method of easily producing a dense colony of mosses from the seedlings in a short period of time.

It has been traditionally preferred that the soil of a 10 garden is covered with various plants to create a natural beauty. A typical example of such soil-covering plants are grass, but mosses are much preferred to grass in the Japanese garden.

Though there are mosses tolerant of a direct sunshine, some species of mosses are weak to a direct sunshine, not adapted 15 for a fertile soil and grow by absorbing moisture in the air through their leaves. Accordingly, attention has recently been paid to the mosses for applications not only in the conventional moss garden, but also in roof garden, etc. Also, they are planted outdoors in hotels, Japanese inns, etc. to create the traditional Japanese 20 atmosphere.

The beauty of the mosses is not originated from flowers unlike floral plants, but it is derived from the dense colony consisting of a large number of individual mosses. However, since the biology of the mosses has been still mostly unknown, it is 25 difficult to cultivate the mosses on a large scale by a usual garden cultivation method. For instance, the cultivation of *Racomitrium canescens* and *Dicranum japonicum* can actually be conducted only by well skilled gardeners.

The conventional cultivation methods of the mosses are generally classified into a transplantation method and a regeneration method.

The transplantation method comprises collecting a 5 desired moss with a soil on which it is growing, and planting it on a desired place. This method is, however, disadvantageous in that it is not suitable for the plantation of the moss in a wide area.

The regeneration method, which utilizes the strong regenerating nature of the mosses, may be classified into a 10 cutting method, a sowing method, a root separation method, etc., and the sowing method is most commonly conducted. In the sowing method, the mat of the moss colony collected in mountains, fields, etc. is separated or divided into each moss plant or small pieces of the moss plant, and planted on a nursery, causing plumules to grow 15 from the separated moss plants or pieces of the divided moss plants, so that they can be multiplied.

However, the sowing method is disadvantageous in that all divided moss plants are rarely regenerated to have plumules, and that even in a case where all divided moss plants are 20 regenerated, it is impossible to sow the divided pieces of the moss plants at a density corresponding to that of the moss colony, resulting in a sparsely grown moss colony at the initial stage. In order to have a dense moss colony, it has to be waited for until lateral buds or gemmae have grown to full size from the 25 subterranean stems. Such growth of the lateral buds or gemmae usually takes several years, during which the moss colony should be well nurtured. Further, in the nursery on a cultivation soil of 30 cm x 60 cm, one can prepare only two to four cases of the nursery a

day, if he works 7 hours a day to sow the divided pieces of the moss plants one by one at a constant interval. This is extremely low efficiency. On the other hand, there are some mosses such as *Polytrichum*, etc. which grow so rapidly that they can form a colony 5 in about one year. Such mosses, however, have so short a life longevity as plants that they die in a short period of time. Because of these reasons, the moss gardens, moss pots, etc. are extremely expensive.

Since mosses such as *Sphagnum* have not only good 10 resistance to fungi but also an excellent water retention ability, they are used to cover soils of pots for expensive and weak floral plants and trees such as orchids, bonsai (dwarfed tree), etc., in order to protect the weak plants and trees from harmful fungi and prevent them from extreme dryness. However, if these mosses are 15 collected in the forests and the mountains excessively, the water retention ability of the forests and the mountains would become so low that landslide, flooding, etc. are likely to take place. Further, once the moss colony is removed from the surface of the soil or rock, it takes many years in a natural environment until the surface 20 of the soil or rock is covered by a moss colony again. Thus, to protect the nature of the earth, it is important to keep the moss colony from being excessively removed from the surface of the soil or rock.

25

Accordingly, an object of the present invention is to provide a moss seedling which can easily grow to a dense moss colony.

Another object of the present invention is to provide a method of producing a moss seedling from which a dense moss colony can easily be formed in a short period of time.

As a result of intense research in view of the above 5 object, the inventor has found that by multiplying plant tissues of mosses such as gametophores, protonemata, spores, calluses, etc. by a pure culture in a culture tank to form seedlings which correspond to plumules, and sowing the seedlings onto a nursery at a desired colony density, a dense moss colony can be formed in a short period 10 of time. The present invention has been completed based on this finding.

Thus, the moss seedling according to the present invention is obtained by pure culture of the plant tissues of the mosses.

15 The method of producing a dense mat of a moss colony according to the present invention comprises the steps of (a) purely culturing the plant tissues of the moss in a culture medium to generate seedlings thereof, (b) sowing the seedlings onto a nursery at a desired colony density, and (c) caring the seedlings so that they 20 mature to full size.

#### [A] Moss seedlings

The mosses (bryophytes) to be cultured to produce 25 seedlings (cultigen) in the present invention generally include musci (Bryopsida), hepaticae (Hepaticopsida) and anthocerotae (Anthocerotopsida). The Bryopsida generally include Sphagnidae, Andreaeidae, Bryidae (Polytrichales, Dicranales, Eubryales, etc.), etc.

The Hepaticae generally include *Marchantiales*, *Metzgeriales*, *Jungermanniales*, etc. The anthocerotae generally include anthocerotaceae, etc.

5 Representative mosses used for moss gardens, moss pots, coverings for flower pots, etc. are *Dicranales*, *Racomitrium*, *Leucobryum* and *Bryum* as well as *Sphagnaceae*, etc.

The seedlings of such mosses will be described below.

First, the plants or spores of mosses are collected in fields or mountains, and the plant tissues of the mosses are 10 multiplied by pure culture to form seedlings. Alternatively, the plant tissues of the mosses may be subjected to pure culture for differentiation to seedlings and then multiplied.

The next point of the present invention is to cultivate the seedlings to form a dense mat of a moss colony. The seedlings 15 to be cultured in the present invention correspond to the plumules. Depending on the plant tissues to be cultured, the seedlings may not be clearly confirmed as the plumules, or the seedlings may be identified only as small projections in the plants. Even in such cases, almost 100% of the plumules can germinate and grow in the 20 method of the present invention.

The plant tissues of the mosses to be cultured may be gametophores, tissue pieces, spores, gemmae, subterranean stems, rhizoids, protonemata, etc. Also, calluses derived from these plant tissues may be cultured.

25 Which forms of plant tissues are most suitable for pure culture and what courses of culture are most suitable depend on the species of the mosses. Further, in some cases the plant tissues

may be treated to derive calluses, which may be then converted to the seedlings such as plumules.

Accordingly, the derivation and culture of the moss seedlings may be conducted by the following methods (A)-(L):

- 5        (A) Gametophores → Seedlings.
- (B) Gametophores → Calluses → Seedlings.
- (C) Gametophores → Protonemata → Seedlings.
- (D) Spores → Protonemata → Seedlings.
- (E) Spores → Protonemata → Calluses → Seedlings.
- 10      (F) Gemmae → Seedlings.
- (G) Gemmae → Calluses → Seedlings.
- (H) Tissue pieces → Seedlings.
- (I) Tissue pieces → Calluses → Seedlings.
- (J) Protonemata → Seedlings.
- 15      (K) Subterranean stems → Seedlings.
- (L) Rhizoids → Seedlings.

The above culture and differentiation of the plant tissues are conducted in a culture medium which may be a solid culture medium or a liquid culture medium. For instance, various Murashige-Skoog modified culture media (MS modified culture media) mentioned below may be selected depending on the plant forms of the mosses to be cultured and the desired forms of the cultured mosses, and if necessary, plant growth control substances such as kinetin, benzyladenine, indole-3-acetic acid, 2,4-dichlorophenoxyacetic acid (2,4-D), etc. may be added.

In general, the plant tissues of the mosses are likely to be converted to calluses in a culture medium having a high

concentration (2 weight % or higher) of monosaccharide such as glucose or disaccharide, and the calluses are easily converted to the seedlings in a culture medium having a low concentration (0.4 weight % or lower) of sugar.

5           The above methods (A)-(L) will be explained in the case of using the MS modified culture media having compositions consisting of Solution Nos. 1-3 (solvent: distilled water) described below; sugars such as glucose; vitamins; organic acids, inorganic acids or their salts; and distilled water.

10    Solution No. 1

	Calcium chloride dihydrate	4,400 mg/liter,
	Magnesium sulfate heptahydrate	3,700 mg/liter,
	Manganese sulfate tetra- to penta-hydrate	223 mg/liter,
	Boric acid	62 mg/liter,
15	Zinc sulfate heptahydrate	107 mg/liter,
	Sodium molybdenate dihydrate	2.5 mg/liter,
	Copper sulfate pentahydrate	0.25 mg/liter,
	Cobalt chloride hexahydrate	0.25 mg/liter, and
	Potassium iodide	8.3 mg/liter.

20

Solution No. 2

Potassium dihydrogenphosphate ( $\text{KH}_2\text{PO}_4$ ) 42.5 mg/liter.

Solution No. 3

25	Myo-inositol	10,000 mg/liter,
	Nicotinic acid	50 mg/liter,
	Pyridoxine-hydrochloric acid	50 mg/liter, and
	Thiamine-hydrochloric acid	10 mg/liter.

Compositions of various MS modified culture media

NA-MS culture medium

	Solution No. 1	100 ml/liter,
5	Solution No. 2	4 ml/liter,
	Solution No. 3	10 ml/liter,
	Glucose	20 g/liter,
	Potassium nitrate	4 g/liter,
	EDTA*-Disodium	47 mg/liter, and
10	Iron sulfate heptahydrate	27.8 mg/liter.
	pH = about 5.8.	

Note: \* Ethylenediaminetetraacetic acid.

AS-MS culture medium

15	Solution No. 1	100 ml/liter,
	Solution No. 2	4 ml/liter,
	Solution No. 3	10 ml/liter,
	Glucose	20 g/liter,
	EDTA*-Disodium	47 mg/liter,
20	Iron sulfate heptahydrate	27.8 mg/liter,
	Ammonium chloride	44 mM, and
	Sodium succinate or sodium fumarate	33 mM.
	pH = about 5.8.	

25 ANA-MS culture medium

	Solution No. 1	100 ml/liter,
	Solution No. 2	4 ml/liter,
	Potassium nitrate	4 g/liter,

EDTA*-Disodium	47 mg/liter, and
Iron sulfate heptahydrate	27.8 mg/liter.
pH = about 5.8.	

5 AAS-MS culture medium

Solution No. 1	100 ml/liter,
Solution No. 2	4 ml/liter,
EDTA*-Disodium	47 mg/liter,
Iron sulfate heptahydrate	27.8 mg/liter,
10 Ammonium chloride	44 mM, and
Sodium succinate or sodium fumarate	33 mM.
pH = about 5.8.	

MSF culture medium

15 Solution No. 1	10 ml/liter,
Solution No. 2	0.4 ml/liter,
Solution No. 3	1 ml/liter,
Glucose	2 g/liter,
Potassium nitrate	0.4 g/liter,
20 EDTA*-Disodium	4.7 mg/liter,
Iron sulfate heptahydrate	2.78 mg/liter,
Ammonium chloride	20 mM, and
Sodium succinate or sodium fumarate	15 mM.
pH = about 5.8.	

25

Culture Method

- (A) Gametophores → Seedlings
- (1) Sterilization

The gametophores are cut to about 1-10 mm, and cultured on a 1/5 (diluted 5 times) NA-MS culture medium (solidified with agar; Gelrite®, etc.) at 20-25°C while illuminating a light of about 1000-3000 lux. After removing portions infected 5 with fungi, etc., the culture operation of the gametophores are repeated several times to produce sterilized gametophores.

The above sterilization operation is conducted, for instance, by washing the gametophores (one type of the plant tissues of the mosses to be sterilized) with a solution (concentration: 10 about 0.1-0.5 volume %) of a nonionic surfactant such as polyoxyethylene sorbitan monolaurate (tradename: Tween 20), polyoxyethylene sorbitan monooleate (tradename: Tween 80), etc. or a cationic surfactant such as benzalkonium chloride, etc. for 40-60 seconds by means of a stainless steel syringe holder, etc.; 15 sterilizing the gametophores with a solution of 0.5-1 volume % of sodium hypochlorite, etc.; and immediately fully washing the gametophores with germ-free distilled water. The gametophores thus sterilized are cultured. If it is found that there are portions in the gametophores infected with the fungi, those portions should be 20 removed, and the above sterilization operation should be repeated until infected portions disappear.

(2) Derivation and multiplication of seedlings

The resulting sterilized gametophores are cultured in a 1/5 ANA-MS culture medium (liquid, photoautotrophic culture 25 medium) in which carbon dioxide is enriched to 1%, or a 1/5 NA-MS culture medium (liquid) containing 0.1-10  $\mu$ M of a growth control substance at 20-25°C for 10-30 days while illuminating a light of

about 1000-3000 lux. As a result, the gametophores give rise to seedlings.

Incidentally, when the growth control substance is added to the culture medium, the derived seedlings are taken out 5 from the culture medium after 3-10 days, fully washed with the 1/5 NA-MS culture medium, and then introduced into the 1/5 NA-MS culture medium containing no growth control substance to continue the culture of the seedlings.

The multiplication of the seedlings can be efficiently 10 conducted, for instance, by pouring the gametophores into a container such as a conical flask, etc. together with a culture medium, and shaking it at 110-120 rpm/min. Alternatively, the gametophores can be poured into a container such as a shallow flask, etc. together with a culture medium to conduct a suspension 15 culture. In the suspension culture, the seedlings derived from the gametophores are cut one after another by shaking, and each divided piece of the seedlings grows. As a result, the sterilized seedlings can be cultured on a large scale.

The seedlings thus cultured are collected from the 20 culture medium, fully washed with a sterilized distilled water to completely remove the culture medium, and then dried at 25-35°C while blowing the air, or under a sunshine, or freeze-dried at a temperature between -20°C and -5°C. Thus, moss seedlings can be obtained in a dry state. The dried seedlings thus obtained can be 25 kept alive in storing for a long period of time (more than 1 year). Alternatively, the moss seedlings dispersed in the culture medium can be retained in a bottle. After multiplication for 4-8 weeks, the

number of the seedlings (cultigen) can be as many as 100-150 times the number of the divided gametophores.

The mosses suitable for this procedure (A) are as follows:

5 (a) Bryopsida

*Atrichum, Fissidens, Blindia, Brachydontium,*  
*Aongstroemia, Brothera, Bruchia, Bryohumbertia, Campylopodium,*  
*Campylopus, Cynodontium, Dichodontium, Dicranella,*  
*Dicranodontium, Dicranoloma, Dicranoweisia, Dicranum, Kiaeria,*

10 *Leucoloma, Oncophorus, Oreas, Oreoweisia, Paraleucobryum,*  
*Rhabdoweisia, Trematodon, Leucobryum, Chenia, Desmatodon,*  
*Dalytrichla, Didymodon, Eucladium, Gymnostomum, Hydrogonium,*  
*Hymenostylium, Hyophila, Leptodontium, Luislerella, Molendoa,*  
*Oxystegus, Phascum, Pottia, Pseudosymbblepharis, Scopelophila,*

15 *Streblotrichum, Timmiella, Tortella, Tortula, Trichostomun,*  
*Tuerckheimia, Weissia, Racomitrium, Rhodobryum, Cinclidium,*  
*Mnium, Orthomnion, Aulacomnium, Pyrrhobryum, Climacium,*  
*Thamnobryum, Dolichomitria, Hookeria, Thamniopsis,*  
*Cyathophorella, Hypopterygium, Thudium, Homalothecium,*

20 *Hypnum*, etc.

(b) Hepaticopsida

*Haplomitrium, Ricciocarpus*, etc.

(B) Gametophores → Calluses → Seedlings

(1) Sterilization

25 The same as in (A).

(2) Derivation of calluses

The gametophores sterilized in the above step (1) are cultured on a 1/5 AS-MS culture medium (solid) in which the

concentration of glucose is controlled to 4-8 weight %, or on an MFS culture medium (solid) at 20-25°C for 30-60 days while illuminating a light of about 1500-2000 lux. As a result, the gametophores give rise to calluses.

5 (3) Multiplication of calluses

The derived calluses are multiplied in an AS-MS culture medium (liquid) in which the concentration of glucose is controlled to 2-4 weight %, or in an AAS-MS culture medium (liquid, photoautotrophic culture medium) in which carbon dioxide is enriched to 1%, at 20-25°C for 15-20 days while shaking at 110-120 rpm/min and illuminating a light of about 1000-3000 lux. After multiplication for 45-90 days, the number of the seedlings (cultigen) can be as many as 100-150 times the number of the divided gametophores.

15 The multiplied calluses may be stored in an AS-MS culture medium in which the concentration of glucose is controlled to 2 weight %.

(4) Derivation of seedlings

The resulting calluses are cultured in a 1/5 ANA-MS culture medium (liquid, photoautotrophic culture medium) in which carbon dioxide is enriched to 1%, or a 1/5 NA-MS culture medium (liquid) at 20-25°C for 30-60 days while shaking at 110-120 rpm/min and illuminating a light of about 1000-3000 lux. As a result, the calluses give rise to seedlings. The seedlings thus obtained may be sown and stored in the same manner as in the above procedure (A). After multiplication for 8-15 weeks, the number of the seedlings (cultigen) can be as many as 100-150 times the number of the divided gametophores.

This procedure is suitable for the mosses which can grow fast through their calluses or those which can be well stored in the state of calluses. Representatives of such mosses are as follows:

5 (a) Bryopsida

*Bartramia, Barbula, Polytrichum, Atrichum, Sphagnum,*  
etc.

(b) Hepaticopsida

*Marchantia, Jungermannia, Plagiochila*, etc.

10 (C) Gametophores → Protonemata → Seedlings

(1) Sterilization

The same as in (A).

(2) Derivation of protonemata

The gametophores sterilized in the above step (1) are

15 cultured on a 1/5 ANA-MS culture medium (solid) at 20-25°C for 5-10 days while illuminating a light of about 1000-3000 lux. As a result, the gametophores give rise to protonemata.

(3) Derivation and multiplication of seedlings

The derived gametophores are multiplied in a 1/5 ANA-MS culture medium (liquid, photoautotrophic culture medium) in which carbon dioxide is enriched to 1%, or in a 1/5 NA-MS culture medium (liquid), at 20-25°C for 30-60 days while shaking at 110-120 rpm/min and illuminating a light of about 1000-3000 lux. As a result, the protonemata give rise to seedlings. After 25 multiplication for 5-10 weeks, the number of the seedlings (cultigen) can be as many as 100-150 times the number of the divided gametophores.

The multiplied seedlings may be stored in the same manner as in the above procedure (A).

This procedure is suitable for the mosses which can grow fast through their protonemata. Representatives of such 5 mosses are as follows:

(a) Bryopsida

*Ceratodon, Distichium, Ditrichum, Bartramia, Piagiopus,*  
etc.

(b) Hepaticopsida

10 *Scapania, Marchantia, Jungermannia*, etc.

(D) Spores → Protonemata → Seedlings

(1) Sterilization

Spores (calyptrae, if any, are removed from capsules) are sterilized in the same manner as in the above procedure (A).

15 (2) Derivation of protonemata

The spores sterilized in the above step (1) are cultured on a 1/5 ANA-MS culture medium (solid) at 20-25°C for 10-15 days while illuminating a light of about 1000-3000 lux. As a result, the spores give rise to protonemata.

20 (3) Derivation and multiplication of seedlings

The same as in (C).

The multiplied seedlings may be stored in the same manner as in the above procedure (A).

This procedure is suitable for the mosses which can 25 grow fast through their spores instead of gametophores.

Representatives of such mosses are as follows:

(a) Bryopsida

*Atrichum, Pogonatum, Polytrichastrum, Polytrichum,  
Dicranum, Leucobryum, Racomitrium, Funaria, Physcomitrium,  
Gymnostomiella, Oedipodium, Splachnum, Tayloria, Tetraplodon,  
Anomobryum, Brachymenium, Bryum, Epipterygium, Leptobryum,  
5 Mielichhoferia, Plagiobryum, Pohlia, Pseudobryum, Rhizomnium,  
Pyrrhobryum, Bartramia, Breutelia, Fleischcerobryum, Philonotis,  
Plagiothecium, Ctenidium, Ectropothecium, Hypnum, etc.*

(b) Hepaticopsida

*Plagiochila, Frullania, Bazzania, etc.*

10 (E) Spores → Protонemata → Calluses → Seedlings

(1) Sterilization

Spores (calyptrae, if any, are removed from capsules) are sterilized in the same manner as in the above procedure (A).

(2) Derivation of protonemata

15 The same as in (D).

(3) Derivation of calluses

The protonemata obtained in the above step (2) are cultured on a 1/5 AS-MS culture medium (solid) in which the concentration of glucose is controlled to 4-8 weight %, or on an MFS

20 culture medium (solid) at 20-25°C for 30-60 days while illuminating a light of about 1500-2000 lux. As a result, the protonemata give rise to calluses.

(4) Multiplication of calluses

The calluses thus derived can be multiplied in the same manner as in the above procedure (B).

(5) Derivation and multiplication of seedlings

The same as in (B).

The multiplied seedlings may be stored in the same manner as in the above procedure (A).

This procedure is suitable for the mosses which can grow fast through their calluses or those which can be well stored 5 in the state of calluses. Representatives of such mosses are as follows:

(a) Bryopsida

*Sphagnum, Barbula, Trachycystis, Bartramia*, etc.

(b) Hepaticopsida

10 *Plagiochila, Radula, Bazzania*, etc.

(F) Gemmae → Seedlings

(1) Sterilization

Gemmae are cut into about 1-10 mm and sterilized in the same manner as in the above procedure (A).

15 (2) Multiplication of gemmae

The sterilized gemmae are multiplied on a 1/5 NA-MS culture medium (solid) at 20-25°C for 10-15 days while illuminating a light of about 1000-3000 lux.

(3) Derivation and multiplication of seedlings

20 The gemmae obtained in the above step (2) are cultured in a 1/5 ANA-MS culture medium (liquid, photoautotrophic culture medium) in which carbon dioxide is enriched to 1%, or in a 1/5 NA-MS culture medium (liquid) containing 0.1-10 µM of a growth control substance, at 20-25°C for 15-30 days while shaking at 110-25 120 rpm/min and illuminating a light of about 1000-3000 lux. As a result, the gemmae give rise to seedlings. After multiplication for 4-8 weeks, the number of the seedlings (cultigen) can be as many as 100-150 times the number of the divided gemmae.

The multiplied seedlings may be stored in the same manner as in the above procedure (A).

This procedure is suitable for the mosses which can grow fast through their gemmae. Representatives of such mosses 5 are as follows:

(a) Bryopsida

*Pohlia, Tetrodontium, Dicranum, Trachycystis*, etc.

(b) Hepaticopsida

*Marchantia, Metzgeria, Conocephalum*, etc.

10 (G) Gemmae → Calluses → Seedlings

(1) Sterilization

The same as in (F).

(2) Derivation of calluses

The gemmae sterilized in the above step (1) are 15 cultured on a 1/5 AS-MS culture medium (solid) in which the concentration of glucose is controlled to 4-8 weight %, or on an MFS culture medium (solid) at 20-25°C for 30-60 days while illuminating a light of about 1500-2000 lux. As a result, the gemmae give rise to calluses.

20 (3) Multiplication of calluses

The calluses thus derived can be multiplied and stored in the same manner as in the above procedure (B).

(4) Derivation and multiplication of seedlings

The calluses can be converted to seedlings and 25 multiplied in the same manner as in the above procedure (B). After multiplication for 8-17 weeks, the number of the seedlings (cultigen) can be as many as 100-150 times the number of the

divided gemmae. The multiplied seedlings may be stored in the same manner as in the above procedure (A).

This procedure is suitable for the mosses which can grow fast through their calluses or those which can be well stored 5 in the state of calluses. Representatives of such mosses are as follows:

(a) Bryopsida

*Hyophila, Pohlia, Pseudotaxiphyllum, Pylaisiadelpha*, etc.

(b) Hepaticopsida

10 *Marchantia, Metzgeria, Cololejeunea*, etc.

(H) Tissue pieces → Seedlings

(1) Sterilization

Plant tissues (collected from stems, leaves, etc.) are cut into about 1-10 mm and sterilized in the same manner as in the 15 above procedure (A).

(2) Derivation and multiplication of seedlings

The plant tissue pieces sterilized in the above step (1) are cultured in a 1/5 ANA-MS culture medium (liquid, photoautotrophic culture medium) in which carbon dioxide is 20 enriched to 1%, or in a 1/5 NA-MS culture medium (liquid) containing 0.1-10  $\mu$ M of a growth control substance, at 20-25°C for 30-60 days while shaking at 110-120 rpm/min and illuminating a light of about 1000-3000 lux. As a result, the plant tissue pieces give rise to seedlings. After multiplication for 5-10 weeks, the 25 number of the seedlings (cultigen) can be as many as 100-150 times the number of the plant tissue pieces.

The multiplied seedlings may be stored in the same manner as in the above procedure (A).

This procedure is suitable for the following mosses:

(a) Bryopsida

*Polytrichum, Leucobryum, Dicranum, Hypnum,*  
*Sphagnum*, etc.

5 (b) Hepaticopsida

*Haplomitrium, Jungermannia, Metzgeria*, etc.

(I) Plant tissue pieces → Calluses → Seedlings

(1) Sterilization

The same as in (H).

10 (2) Derivation of calluses

The plant tissue pieces sterilized in the above step (1) are cultured on a 1/5 AS-MS culture medium (solid) in which the concentration of glucose is controlled to 4-8 weight %, or on an MFS culture medium (solid) at 20-25°C for 30-60 days while

15 illuminating a light of about 1500-2000 lux. As a result, the plant tissue pieces give rise to calluses.

(3) Multiplication of calluses

The calluses thus derived can be multiplied and stored in the same manner as in the above procedure (B).

20 (4) Derivation and multiplication of seedlings

The calluses can be converted to seedlings and multiplied in the same manner as in the above procedure (B). After multiplication for 7-12 weeks, the number of the seedlings (cultigen) can be as many as 100-150 times the number of the

25 plant tissue pieces. The multiplied seedlings may be stored in the same manner as in the above procedure (A).

This procedure is suitable for the following mosses:

(a) Bryopsida

*Atrichum, Polytrichum, Barbula, Sphagnum, etc.*

(b) Hepaticopsida

*Plagiochila, Riccia, etc.*

(J) Protonemata → Seedlings

5 (1) Sterilization

Protonemata are cut into about 1-10 mm and sterilized in the same manner as in the above procedure (A).

(2) Multiplication of protonemata

The sterilized protonemata are multiplied on a 1/5 NA-  
10 MS culture medium (solid) at 20-25°C for 5-10 days while illuminating a light of about 1000-3000 lux.

(3) Derivation and multiplication of seedlings

The protonemata thus obtained are cultured in a 1/5 ANA-MS culture medium (liquid, photoautotrophic culture medium)  
15 in which carbon dioxide is enriched to 1%, or in a 1/5 NA-MS culture medium (liquid) containing 0.1-10  $\mu$ M of a growth control substance, at 20-25°C for 30-60 days while shaking at 110-120 rpm/min and illuminating a light of about 1000-3000 lux. As a result, the protonemata give rise to seedlings. After multiplication  
20 for 6-10 weeks, the number of the seedlings (cultigen) can be as many as 100-150 times the number of the divided protonemata.

The multiplied seedlings may be stored in the same manner as in the above procedure (A).

This procedure is suitable for the following mosses:

25 (a) Bryopsida

*Ditrichum, Ceratodon, Bartramia, etc.*

(b) Hepaticopsida

*Marchantia, Scapania, Jungermannia, etc.*

(K) Subterranean stems → Seedlings

(1) Sterilization

Subterranean stems are cut into about 1-10 mm and sterilized in the same manner as in the above procedure (A).

5 (2) Derivation and multiplication of seedlings

The subterranean stems sterilized in the above step (1) are cultured in a 1/5 ANA-MS culture medium (liquid, photoautotrophic culture medium) in which carbon dioxide is enriched to 1%, or in a 1/5 NA-MS culture medium (liquid) containing 0.1-10  $\mu$ M of a growth control substance, at 20-25°C for 10-30 days while shaking at 110-120 rpm/min and illuminating a light of about 1000-3000 lux. As a result, the subterranean stems give rise to seedlings. After multiplication for 3-4 weeks, the number of the seedlings (cultigen) can be as many as 100-150 times the number of the divided subterranean stems.

The multiplied seedlings may be stored in the same manner as in the above procedure (A).

This procedure is suitable for the following mosses:

(a) Bryopsida

20 *Rhodobryum*, *Hylocomium*, *Climacium*, *Pleuroziopsis*, etc.

(L) Rhizoids → Seedlings

(1) Sterilization

Rhizoids are cut into about 1-10 mm and sterilized in the same manner as in the above procedure (A).

25 (2) Derivation and multiplication of seedlings

The rhizoids sterilized in the above step (1) are cultured in a 1/5 ANA-MS culture medium (liquid, photoautotrophic culture medium) in which carbon dioxide is enriched to 1%, or in a 1/5 NA-

MS culture medium (liquid) containing 0.1-10  $\mu\text{M}$  of a growth control substance, at 20-25°C for 10-30 days while shaking at 110-120 rpm/min and illuminating a light of about 1000-3000 lux. As a result, the rhizoids give rise to seedlings. After multiplication for 5 3-4 weeks, the number of the seedlings (cultigen) can be as many as 100-150 times the number of the divided rhizoids.

The multiplied seedlings may be stored in the same manner as in the above procedure (A).

This procedure is suitable for the following mosses:

10 (a) Bryopsida

*Racomitrium, Dicranum, Ptilium, Thuidium*, etc.

(b) Hepaticopsida

*Haplomitrium, Bazzania, Marchantia*, etc.

15 Though the moss seedlings and the method of producing a dense moss mat from these seedlings have been described above together with specific examples of mosses suitable for such applications, it should be noted that the present invention is not limited to them. Any other culture media commonly used for pure 20 culture may be conveniently utilized in the present invention. Also, the formation of calluses can be conducted, if necessary, for the purpose of multiplication and storing. For instance, into the culture method containing no callus-forming step, the callus-forming step may be introduced. In addition to the above-described culture 25 methods, special culture methods may be used. For instance, in the case of *Schistostega*, the above procedure (D) may be stopped at the stage of multiplication of protonemata, and the multiplied protonemata may preferably be sown as seedlings.

The seedlings obtained in each of the above procedures (A)-(K) may be charged into a culture vessel again to produce the seedlings of the same quality (clones of mosses) repeatedly by pure culture.

##### 5 [B] Production of dense moss mat

The method of producing a dense moss mat using the seedlings according to the present invention will be explained below.

First, the seedlings obtained by the above procedures  
10 are sown onto a nursery. The sowing density may be determined depending on the desired colony density. In general, in the case of a moss garden, the seedlings are sown at a density of about 5-20 per 1 cm<sup>2</sup>. Also in the case of the dried seedlings, they may be sown directly onto the nursery or after immersion in a water.

15 Further, in a case where the seedlings are kept in a culture medium, they should be washed with water and then sown. Moisture need not be applied under usual conditions, but when the atmosphere and the nursery are dry, water may be preferably supplied onto the nursery by spraying, etc. such that the nursery  
20 becomes slightly moist.

The nursery is generally produced from Hyuga soil, Kanuma soil, vermiculite, peat moss, leaf mold, river sand, etc. alone or in combination. Further, foamed perlite (tradename: Aquasoil, etc.), water-absorbing polymers (tradename: Sumikagel, etc.), etc.  
25 may be used. In addition, a small amount of charcoal powder may be added. When the sand soil is used for the nursery, the sand soil is preferably sterilized by boiling, baking in an oven, etc.

The sowing of the seedlings may be conducted directly onto a final cultivation place such as a moss garden, a moss pot, etc., but it is preferable that the seedlings are first sown onto a nursery pallet of about 10-30 cm x 20-60 cm and then transplanted to the 5 final cultivation place after a relatively dense moss colony is formed. Also, in the case of a large-scale cultivation, the seedlings may be sown directly onto a large cultivation place, or a large number of nursery pallets may be used to cultivate the moss seedlings.

10 After sowing, the seedlings are cared for 150-240 days. For instance, in the case of *Raconitrium canescens*, it forms a relatively dense colony 6 months after the sowing. To obtain a green and healthy moss colony, it is preferable to care the moss in a sunshine while being in contact with the atmosphere. On the other 15 hand, if the moss is used in an application in which appearance is not an important factor (for instance, for sterilization of soil in flower pots or bonsai), the care of the moss grown from the seedlings may be conducted by placing the nursery pallets in plastic greenhouses or casings to avoid the influence of wind and 20 rain. In the latter case, the moss becomes pale green and susceptible to the change of environment, though it can be grown surely and rapidly.

After the moss grows sufficiently, it is transplanted to a final cultivation place such as a moss garden, a moss pot, etc., and 25 kept for 10-15 days so that the moss colony becomes stable on the soil.

Incidentally, in the above cultivation process, when the moss grows non-uniformly to form a moss colony having a non-

uniform density, the cull-out or transplantation of moss is necessary to make the density of the moss colony uniform.

In the conventional moss-sowing method, it takes about 30-60 days until plumules are formed on moss pieces sown onto the soil, and it is rare that substantially all moss pieces have plumules. In addition, even if substantially all the moss pieces have plumules, the resulting moss colony fails to have a high density. Accordingly, at the initial stage of forming the moss colony, it is very sparse, and it must be waited for until lateral buds 10 grown from the subterranean stems and the rhizoids propagate sufficiently if the moss colony is to be made uniform. This usually takes 800-1100 days. For instance, in the case of *Dicranum scoparium*, it takes 900-1000 days. This means that it takes an extremely long time until a sufficiently dense moss colony can be 15 obtained by the conventional moss-sowing method.

However, in the present invention, the mosses are purely cultured under the most suitable conditions to produce their seedlings on a large scale in an extremely short period of time. Since the cultured seedlings can surely germinate, a dense moss 20 colony can be formed in a greatly reduced period of time by sowing these cultured seedlings onto a nursery.

In the cultivation method of the present invention, it takes only about 200-300 days until the growing of the moss is completed after the initiation of pure culture, though the time 25 period may vary within the above range depending on the types of the mosses. For instance, in the case of *Dicranum scoparium*, it takes about 210-240 days, which is much shorter than in the conventional moss-sowing method.

Further, in the conventional moss-sowing method, divided leaves and stems of mosses are manually sown one by one onto a nursery. On the other hand, since the seedlings of the present invention are kept in a dry state or in a culture medium, 5 sowing can be completed only by scattering the seedlings at a high density onto a nursery. Therefore, the sowing operation is extremely easy and versatile in the present invention, promising high efficiency.

The present invention will be explained in detail by way 10 of the following Examples without intention of restricting the scope of the present invention thereto.

Example 1

The plant bodies of *Dicranum scoparium* were collected, and only gametophores were separated out, cut to about 5 mm by a 15 scissors and sterilized. Each one of the cut gametophores was cultured on a 1/5 NA-MS culture medium (solidified with 8% agar) at 25°C while illuminating a light of 2000 lux. After removing portions infected with fungi, etc., the culture operation of the 20 gametophores were repeated 5 times to produce sterilized gametophores.

The sterilized gametophores were then charged together with 1  $\mu$ M of kinetin (growth control substance) into a flask containing a 1/5 ANA-MS culture medium (liquid) in which carbon dioxide was enriched to 1%, and the flask was set in a shaking 25 machine. The culture of the gametophores was conducted at 25°C for 7 days while shaking at 110 rpm/min and illuminating a light of 2000 lux, so that the gametophores were differentiated to form seedlings.

The seedlings thus cultured were removed from the flask, fully washed with the 1/5 NA-MS culture medium, and then introduced into the 1/5 NA-MS culture medium containing no growth control substance to continue the culture of the seedlings 5 under the same conditions for 6 weeks. As a result, about 100 times as many seedlings as the original cut and sterilized gametophores were obtained. The seedlings were fully washed with a sterilized distilled water to completely remove the culture medium, and then dried at 30°C while blowing the air. Thus, the 10 moss seedlings were obtained in a dry state.

The seedlings were sown at a density of about 5 per 1 cm<sup>2</sup> onto a nursery pallet of 30 cm x 60 cm x 3 cm filled with sand in a thickness of 1 cm, and then cultivated in an open place.

After 180 days, the *Dicranum scoparium* formed a 15 dense moss colony, which could be transplanted to a final cultivation place such as a moss garden.

#### Comparative Example 1

A colony of *Dicranum scoparium* was manually separated to individual stems, and they were sown onto a nursery 20 at a density of 30 x 30 per a pallet in the same manner as in Example 1.

The *Dicranum scoparium* grew extremely slowly as compared with those in Example 1, and the resulting moss colony was still sparse after 180 days, failing to be transplanted to a final 25 cultivation place.

#### Example 2

Sporophytes of *Pyrrhobryum dozyanum* were collected, and only capsules were separated out with calyptae removed to

obtain spores. The spores were cultured on a 1/5 NA-MS culture medium (solidified with 8% agar) at 25°C while illuminating a light of 2000 lux. After removing portions infected with fungi, etc., the culture operation of the gametophores were repeated 5 times to 5 produce sterilized spores.

The sterilized spores were then cultured on a 1/5 NA-MS culture medium (solidified with 8% agar) at 25°C for 14 days while illuminating a light of 2000 lux. As a result, protonemata were obtained.

10 The protonemata were charged together with 1  $\mu$ M of kinetin (growth control substance) into a flask containing a 1/5 ANA-MS culture medium (liquid) in which carbon dioxide was enriched to 1%, and the flask was set in a shaking machine. The culture of the protonemata was conducted at 25°C for 7 days while 15 shaking at 110 rpm/min and illuminating a light of 2000 lux, so that the protonemata were differentiated to form seedlings.

The seedlings thus cultured were removed from the flask, fully washed with the 1/5 NA-MS culture medium, and then introduced into the 1/5 NA-MS culture medium containing no 20 growth control substance to continue the culture of the seedlings under the same conditions for 50 days. The seedlings were fully washed with a sterilized distilled water to completely remove the culture medium, and then dried at 30°C while blowing the air. Thus, the moss seedlings were obtained in a dry state.

25 The seedlings were sown at a density of about 5 per 1  $\text{cm}^2$  onto a nursery pallet of 30 cm x 60 cm x 3 cm filled with sand in a thickness of 1 cm, and then cultivated in an open place.

After 180 days, the *Pyrrhobryum dozyanum* formed a dense moss colony, which could be transplanted to a final cultivation place such as a moss garden.

Example 3

5 Sporophytes of *Barbula unguiculata* were collected, and only capsules were separated out and sterilized with calyptrae removed to obtain spores. The spores were cultured on a 1/5 NA-MS culture medium (solidified with 8% agar) at 25°C while illuminating a light of 2000 lux. After removing portions infected  
10 with fungi, etc., the culture operation of the gametophores were repeated 5 times to produce sterilized spores.

The sterilized spores were then cultured on a 1/5 NA-MS culture medium (solid) at 25°C for 14 days while illuminating a light of 2000 lux. As a result, protonemata were obtained.

15 The protonemata were cultured on a 1/5 AS-MS culture medium (solid) in which the concentration of glucose was controlled to 4 weight % at 25°C for 50 days while illuminating a light of 1500 lux. As a result, calluses were obtained.

The calluses were charged together with 1  $\mu$ M of  
20 kinetin (growth control substance) into a flask containing a 1/5 ANA-MS culture medium in which carbon dioxide was enriched to 1%, and the flask was set in a shaking machine. The culture of the calluses was conducted at 25°C for 50 days while shaking at 110 rpm/min and illuminating a light of 2000 lux, so that the calluses  
25 were differentiated to form seedlings.

The seedlings thus cultured were removed from the flask, fully washed with the 1/5 NA-MS culture medium, and then introduced into the 1/5 NA-MS culture medium containing no

growth control substance to continue the culture of the seedlings under the same conditions for 50 days. The seedlings were fully washed with a sterilized distilled water to completely remove the culture medium, and then dried at 30°C while blowing the air.

5 Thus, the moss seedlings were obtained in a dry state.

The seedlings were sown at a density of about 5 per 1 cm<sup>2</sup> onto a nursery pallet of 30 cm x 60 cm x 3 cm filled with sand in a thickness of 1 cm, and then cultivated in an open place.

10 After 180 days, the *Barbula unguiculata* formed a dense moss colony, which could be transplanted to a final cultivation place such as a moss garden.

15 As described in detail, since plant tissues of mosses such as gametophores, calluses, protonemata, etc. are purely cultured in the present invention, they can be multiplied on a large scale in a culture tank to produce seedlings corresponding to plumules in a short period of time. The seedlings (cultigen) may be sown onto a nursery to form a dense moss colony. By this procedure, the dense moss colony can be easily produced in a greatly reduced period of time as compared with the conventional methods.

20 Also, according to the cultivation method of the present invention, the seedlings corresponding to plumules can be sown at a desired density, so that manual operations which are inevitable in the conventional methods can be eliminated.

25 The method of the present invention makes it possible to produce dense moss mats of various mosses at a high density in a short period of time.

**CLAIMS:**

1. A moss seedling obtained by sterile culture of plant tissues of said mosses.
2. A moss seedling according to claim 1, wherein said plant tissues are cut tissue pieces, gametophores, calluses, protonemata, spores, gemmae, subterranean stems or rhizoids.
3. A moss seedling according to claim 1 or 2, wherein said seedling is kept in a dry state.
4. A moss seedling according to claim 1 or 2, wherein said seedling is kept in culture medium.
5. A process for producing moss seedlings as claimed in claim 1 wherein plant tissues of said moss are subjected to sterile culture to generate seedlings thereof.
6. A process according to claim 5, said process comprising the generation of seedlings from cut tissue pieces, gametophores, calluses, protonemata, spores, gemmae, subterranean stems or rhizoids.
7. A process according to claim 5 or 6 wherein the culture medium contains 4-8% (w/v) of a mono- or di-saccharide for the generation of calluses from plant tissue.
8. A process according to claim 5 or 6 wherein the culture medium contains 2-4% (w/v) of a mono- or di-saccharide for the multiplication of calluses.

9. A process according to claim 5 or 6 wherein the culture medium contains 0.4% (w/v) of a mono- or di-saccharide for the generation of seedlings from calluses.
10. A process according to any of claims 5 to 9, wherein said seedlings are subsequently subjected to drying.
11. A process according to any of claims 5 to 9, wherein said seedlings are subsequently kept in culture medium.
12. A process for producing a dense moss mat comprising the steps of (a) sterile culture of plant tissues of said mosses in a culture medium to generate seedlings thereof according to any of claims 1 to 4, (b) sowing of said seedlings onto a nursery at a desired colony density, and (c) caring for said seedlings so that they mature to full size.
13. A moss seedling as claimed in claim 1 substantially as herein described with reference to the Examples.
14. A process as claimed in claim 5 substantially as herein described with reference to the Examples.

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 Examiner's report to the Comptroller under Section 17  
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Relevant Technical Fields		Search Examiner C SHERRINGTON
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<b>Databases (see below)</b> (i) UK Patent Office collections of GB, EP, WO and US patent specifications. (ii) ONLINE DATABASES: WPI, CLAIMS, DIALOG/BIOTECH		Documents considered relevant following a search in respect of Claims :- 1-14

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Category	Identity of document and relevant passages		Relevant to claim(s)
A	GB 1401665	(THE BRITISH PETROLEUM COMPANY LIMITED) whole document especially page 1, lines 91 to 93; Examples 1 to 3	1-14
A	EP 0249772 A2	(CPC INTERNATIONAL INC) whole document	1-14
A	EP 0281374 A2	(MITSUI PETROCHEMICAL INDUSTRIES LTD) whole document, especially page 4, lines 10 to 11; Examples	1-14

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